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USAMRMC ltr, 27 Feb 2003

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Award Number: DAMD17-97-1-7001

TITLE: The Symptomatic Persian Gulf Veterans Protocol: An Analysis of Risk Factors with an Immunologic and Neuropsychiatric Assessment

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REPORT DATE: January 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 2001	Annual (23 Dec 96 - 22 Dec 00)

4. TITLE AND SUBTITLE
The Symptomatic Persian Gulf Veterans Protocol: An Analysis of Risk Factors with an Immunologic and Neuropsychiatric Assessment

5. FUNDING NUMBERS
DAMD17-97-1-7001

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, Jan 01). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Veterans returning from the Persian Gulf reported a myriad of complaints. Several hypothesis have been generated to account for these complaints and include exposure and infection with mycoplasma or related organisms and alterations in immunological responsiveness. To investigate these possiblities, we are studying Gulf War Veterans who have complaints referable to at least 2 organ systems that have been otherwise unexplained. Control groups include in theatre veterans who are asymptomatic and non-theatre veterans who are applying for disablity. We have identified 177 symptomatic veterans, 193 asymptomatic controls, and 312 disability controls. We collected samples from 52 of the symptomatic controls, 31 asymptomatic controls, and 21 of the disability controls. Evaluation of peripheral blood mononuclear cells, urine and throat cultures samples for the presence of mycoplasma or ureaplasma organisms by culture and PCR revealed no discernable significant differences. Similarly, no significant differences have been detected in *in-vitro* responsiveness of measures of cell and humoral immunity.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Gulf War Syndrome	37
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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TABLE OF CONTENTS:

5. Introduction:

The overall project objective is to determine if there are fundamental immunologic abnormalities in Gulf War veterans which might be associated in a case control study with their service in the Gulf. In order to determine this, the following specific goals will be addressed: 1) evaluate Persian Gulf Veterans with symptoms to determine if there are *in vitro* abnormalities of immunologic tests; 2) evaluate Persian Gulf War veterans to determine if there is evidence to suggest increased exposure to mycoplasma.

6. Body:

This study will determine whether specific *in-vitro* immunological abnormalities or evidence of differing exposure to mycoplasma can be detected in GW participants who are symptomatic, but in whom no specific diagnosis has as yet been made. As controls, we are assessing GW veterans who are asymptomatic and non-theater veterans who have applied for disability.

To date: 1,541 Persian Gulf War veterans have been evaluated in this study who were also enrolled in the Birmingham registry. Charts have been available for 1,347 and they have been carefully reviewed and have had second physician review. One hundred and seventy-seven have illnesses involving more than two organ systems that have not been accounted for with a defined illness. Samples have been obtained from 52 of these.

Second, we have received and have categorized the list of veterans who served in the Persian Gulf from the Birmingham catchment area. This is the group of individuals who will represent our in-theater, asymptomatic individuals and will be drawn from a list of 1,383 in theater Alabama veterans. There are 193 in the Birmingham area, of whom we have evaluated 31 samples.

Third, we identified individuals in the Birmingham catchment area who were not in theater, but who have applied for disability. These individuals represent the second control group we have identified as a listing of 312 veterans and we have obtained samples from 21.

Patients' demographics are further described in the appended manuscript entitled "*In* vitro Immunological Measures are not altered in symptomatic Gulf War Veterans."

Peripheral blood mononuclear cells, throat swabs and urines have been evaluated for the presence of mycoplasma or ureaplasma. Methodology is described in the attached manuscript "Genotypic and phenotypic analysis of M. fermentans strains isolated from different host tissues". Each sample was evaluated by a) direct culture; b) direct PCR for M. fermentans; and c) by PCR with a universal mycoplasma primer. Mycoplasma or ureaplasma were detected frequently in urine and throat swabs, however no significant differences have been noted between the three groups

Data are expressed as number positive/evaluable samples.

SAMPLE	Asymptomatic	Symptomatic	Disability
PB mononuclear cells			
Culture	0/28	0/47	0/18
Direct PCR	0/30	2/51	0/15 (3 inh)

PCR with primer	0/30	0/49 (2inh)	0/19
Throat			
Culture	14/29	16/51	20/21
Direct PCR	1/29	1/47	3/21
PCR with primer	20/29	41/50	7/21
Urine			
Culture	7/29 *	13/51 **	8/21 ***
Direct PCR	0/29	0/51	0/21
PCR with primer	4/29	5/49 (2 inh)	2/21

Inh-indicated the number of samples in each group which contained an inhbitor which precluded PCR. Each of these samples was run at least twice to confirm the presence of an inhibitor.

*-cultures were positive in 7 samples and each contained ureaplasma urealyticum. Four samples also contained mycoplasma species.

**-cultures were positive in 13. 12 contained ureaplasma urealyticum, 1 contained mycoplamsa alone and 5 contained ureaplasma and mycoplasma species.

***-Cultures were positive in 8. All 8 contained ureaplasma urealyticum and 3 also contained mycoplasma species.

Testing has been completed on the cohort of Gulf War veterans and controls for the a number of *in vitro* immunological functions and plasma assays of certain cytokines and related molecules. Details of this methodology and results are included in the appended manuscript entitled "*In* vitro Immunolgical Measures are not altered in symptomatic Gulf War Veterans."

7. Key Research Accomplishments:

-Our study has determined that there are not any significant differences between symptomatic GW veterans when compared to controls in terms of evidence of exposure to mycoplasma

-Our study has determined that there are no significant abnormalities in *in-vitro* tests of immunologic function or of plasma levels of certain cytokines and related molecules including IL-1β, IL-4, IL-6, IL-10, IFNα, sIL-2R, and sICAM-1. documented any significant differences in *in-vitro* immunological testing in symptomatic GW veterans when compared to controls.

-Overall *in*-vitro responsiveness to anthrax was low in each group when compared to responsiveness to tetanus. Moreover, response to anthrax was lower in the two symptomatic groups when compared to asymptomatic Gulf War Veterans.

8. **Reportable Outcomes:**

1. Campo L. Larocque P. La Malfa T. Blackburn WD. Watson HL. Genotypic and phenotypic analysis of Mycoplasma fermentans strains isolated from different host tissues. Journal of Clinical Microbiology. 36(5):1371-7, 1998.
2. Everson MP, Kotler S, and Blackburn WD. Stress and Immune Dysfunction in Gulf War Veterans Annals NY Academy of Science 876: 413-418, 1999.
3. *Is There Immune Dysregulation in Symptomatic Gulf War Veterans? Michael P. Everson, Ke Shi, Peggy Aldridge, Alfred A. Bartolucci, and Warren D. Blackburn, Jr. VA Medical Center and UAB, Birmingham, Alabama, USA, 1999. *Presented as an abstract in Germany, summer 1999:
4. Everson, M.P., Shi, K. Aldridge, P., Bartolucci A., and Blackburn, WD. Is there immune dysregulation in symptomatic Gulf War Veterans. Z. rheumatol 59: S2, 124-126, 2000

9. **Conclusions:**

The observations from these studies demonstrate that:

1. There were no significant differences between symptomatic Gulf War Veterans and our 2 control groups in the prevalence of detection of mycoplasma in peripheral blood mononuclear cells, throat swabs, and urine.
2. *Ex-vivo* immunological testing evaluating function of accessory cells, T cells, Th1 and Th2 cells, and B cells did not demonstrate any significant depletion or dysfunction when these cells were isolated from symptomatic Gulf War Veterans.
3. *Ex-vivo* immunological testing as described in conclusion 2 did not demonstrate any significant differences in cells obtained from symptomatic Gulf War Veterans when to with cells from asymptomatic Gulf War Veterans and disability controls.

10. **References:** N/A.

JOURNAL OF CLINICAL MICROBIOLOGY, May 1998, p. 1371–1377 Vol. 36, No. 5
0095-1137/98/$04.00+0

Genotypic and Phenotypic Analysis of *Mycoplasma fermentans* Strains Isolated from Different Host Tissues

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Received 17 December 1997/Returned for modification 22 January 1998/Accepted 18 February 1998

A correlation was found between the expression of a specific *Mycoplasma fermentans* surface antigen (Pra, proteinase-resistant antigen) and the site of isolation of the organism from the infected host. Strains which expressed Pra were most frequently associated with cells of bone marrow origin, and strains which lacked expression of Pra were most commonly isolated from the respiratory tract, genital tract, and arthritic joints, i.e., epithelial cell surfaces. Pra was previously shown to be resistant to degradation by proteinases and was hypothesized to play a protective role at the organism surface and perhaps to influence which host tissue site was colonized by the organism. The methods used for this phenotyping scheme required isolation and growth of the mycoplasma in quantities sufficient for immunoblot analysis using monoclonal antibodies. We wanted to determine a more rapid and less cumbersome technique to supplement this method for determining the Pra phenotype directly in clinical specimens. Here we describe PCR studies to investigate the movement of a previously identified *M. fermentans* insertion sequence (IS)-like element. These data showed a correlation between a specific IS genotype and the Pra$^+$ phenotype. Production of a 160-bp product using a single set of IS-based primers was associated with expression of Pra. The genomic IS location resulting in the 160-bp product was determined by using Southern blot analysis and was found to be a stable insertion site characteristic of genotype I strains. Additional analyses of sequences within and flanking the IS insertion sites revealed another pair of PCR primer sites which resulted in the consistent production of a 450-bp amplicon. The stability of this site was dependent on the absence of the IS-like element between the primer sites. The production of this 450-bp amplicon correlated with the Pra mutant phenotype and was characteristic of genotype II strains. The data showed that the sequence within the IS may be unstable and that reliable genotyping sequences are more easily found in the stable genomic sites which flank the IS element.

First mistakenly identified as a novel AIDS-associated virus (18), *Mycoplasma fermentans* incognitus, during the ensuing years, was considered to be a possible cofactor contributing to acceleration of the progression of this immune disorder (8, 16, 20–22, 29). Immediately following the first reports, several laboratories began probing into this question, but to date, the hypothesis of a mycoplasma-AIDS association remains unproved. However, these studies have added much to our basic knowledge of mycoplasmas. It has been documented that *M. fermentans*, as well as some other mycoplasmas, can occur intracellularly, which was only an occasionally reported and unproved observation prior to these studies. The ability of specific subpopulations of these organisms to survive within host cells could account, at least in part, for the characteristic chronicity of mycoplasmal disease, as well as for the frequent difficulty of isolation by culture. Additionally, an impressive volume of literature is accumulating which describes the induction of various cytokines by mycoplasma infection (3, 5, 15, 27, 28, 30, 37). The potential to alternately stimulate or suppress the immune system would impart a distinct advantage to any pathogen (or commensal organism) attempting to survive in the hostile and changing environment of an infected host.

Subsequent to the initial isolation of strain incognitus, *M. fermentans* was identified as the likely etiologic agent of an acute fatal disease in otherwise healthy adults (17). No other infectious agents were found. A similar wasting syndrome leading to death was reported in silvered leaf monkeys after experimental infection with this same agent (19). Many years prior to these recent studies, *M. fermentans* was isolated from bone marrow of leukemic patients (24) and other reports associated it with rheumatoid arthritis (2, 36). These reports prompted further investigations, including some experimental studies with animal models (9, 10, 26). None of these studies resulted in data proving a cause-and-effect relationship between *M. fermentans* infection and human disease. In fact, early serologic studies provided evidence that antibodies to this organism are common in adolescents and young adults (32).

Therefore, *M. fermentans* has been tentatively associated with disease throughout its history but the precise etiologic role of *M. fermentans* in disease remains unclear. This is, in part, due to the frequently unsuccessful attempts to isolate mycoplasmas in general by routine culture methods (6) and to the presence of individuals harboring the organism without signs of disease. Even though many cases have resulted in isolation of *M. fermentans* and each isolate has been assigned a new strain designation, there has been no attempt to assign molecular or functional characteristics to these strains which might assist in determining if there is a characteristic or group of characteristics which associate with specific diseases, or at least with sites of isolation.

In the present study, we were interested in defining methods to determine if specific strains exhibit characteristics which are more frequently associated with particular tissue sites within an infected host. We tested whether monoclonal antibodies (MAbs) developed against *M. fermentans* antigens could dis-

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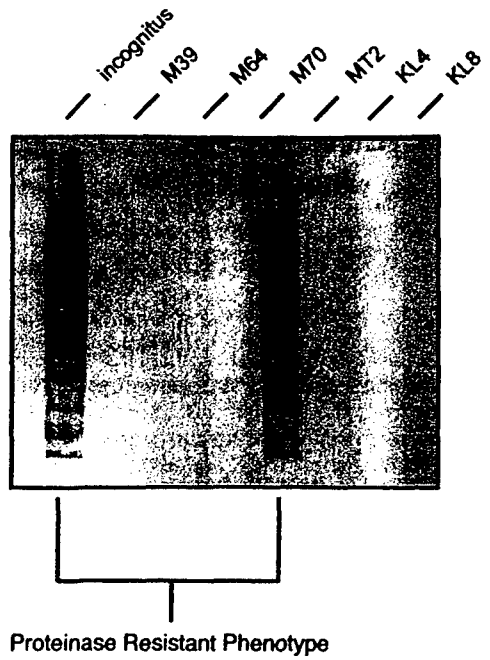

FIG. 1. Pra phenotypes of representative *M. fermentans* strains. Organism proteins were separated by SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose. Reactivity with MAb 1A2.6 was visualized by using peroxidase conjugates. Strains incognitus and MT2 show the characteristic Pra^+ pattern, while the Pra^- strains show no reaction with the MAb. Results for all strains are summarized in Table 1.

tinguish between isolates of *M. fermentans* to determine a possible correlation between the expression of these factors and the site of isolation. We also conducted the same correlative assessment for the chromosomal distribution of the *M. fermentans* insertion sequence (IS)-like element, hypothesizing a role for this potentially mobile element in the repression or activation of a specific gene expression.

MATERIALS AND METHODS

Sources of isolation. The *M. fermentans* strains evaluated in this study were isolated from various sources (see Table 1). Strains were obtained as follows: E10 (24) and K7 (25) were obtained from W. H. Murphy; 16700, 12406, and DEPB were from the University of Alabama at Birmingham; AOU was from Luc Montagnier (Pasteur Institute, Paris, France); Z62 was from P. Hannan (Beecham Labs) (24); incognitus was from Shyh Lo (National Institute of Allergy and Infectious Diseases [NIAID]) (17, 18, 21); AMSO was from Ann Robinson (Laboratory of Immune Genetics, NIAID); MT2 was from W. J. Leonard and N. F. Halden (National Institute of Child Health and Human Development) (11); Elliman was from H. Elliman (University of Illinois, Chicago); 48429 was from Andy Lewis (NIAID); M51, M39, M52, M64, M73, and M70 were from R. Dular (Public Health Laboratory, Ottawa, Ontario, Canada); KL4 and KL8 were from P. Hannan (Beecham Labs); and PG18 was from Klieneberger-Nobel, Lister Institute, London, United Kingdom.

Organisms and growth conditions. Cultures of *M. fermentans* were grown in SP-4 medium (mycoplasma broth base, tryptone [Difco], peptone [Difco], arginine, phenol red [1%], DNA, and antibiotics for SP-4, supplemented with 10% fetal bovine serum, CMRL 1066, yeast extract, yeastolate, and glucose). The cultures were incubated at 37°C. Samples were harvested and washed in phosphate-buffered saline at pH 7.3. DNA was purified in accordance with standard protocols (phenol-chloroform-isoamyl alcohol) and concentrated by ethanol precipitation. DNA preparations were RNase A treated (Sigma).

Southern blotting and DNA hybridization. For Southern blotting of the *M. fermentans* strains, 0.2 μg of genomic DNA was digested with 5 U of *Hin*dIII (Promega, Madison, Wis.) for 2 h at 37°C. The samples were electrophoresed on 0.8% Tris-borate-EDTA agarose gels (50 V for 16 h) and transferred to 1× Hybond N^+ nylon membranes. DNA was UV cross-linked to the membrane in a UV Stratalinker 1800 (Stratagene). After prehybridization, the membranes were hybridized with 5′-end γ-^{32}P-labeled oligonucleotide probe RW006 (5′-GCT GTG GCC ATT CTC TTC TAC GTT-3′; see Fig. 3a) and probe ORF-1 (5′-GGA AAA CTC TTA TTC AGC C-3′; see Fig. 3b), located within the insertion sequence transposase gene and open reading frame 1 (ORF-1). Hybridization was performed at 42°C for 1 h in Rapid hyb (Amersham) hybridization buffer and followed by one washing in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) for 20 min at room temperature and two washings in 0.5× SSC–0.1% SDS for 15 min each at 45°C. DNA hybrids were visualized by autoradiography using Kodak XAR-5 film (Eastman Kodak Co., Rochester, N.Y.).

PCR. After incubation and sufficient growth, the 21 strains were processed with proteinase K combined with buffer A (1 M Tris-HCl [pH 8.0], 1 M KCl, 1 M $MgCl_2$, Milli-Q distilled H_2O) and buffer B (1 M Tris-HCl [pH 8.0], 1 M $MgCl_2$, Triton X-100, Tween 20, Milli-Q distilled H_2O). One milliliter of culture was centrifuged for 20 min at 4°C, the supernatant was discarded, and the pellet was resuspended in proteinase K lysis buffer. The samples were incubated at

TABLE 1. Relationships among the genotypes, phenotypes, and sources of isolation of different *M. fermentans* strains

Strain[a]	PCR product size (bp)[b]	Genotype determined by Southern blotting[c]	Immunoblotting result[d]	Specimen origin
M51	Diffuse	II	−	Respiratory tract
M39	80	II	−	Respiratory tract
M52	Diffuse	II	−	Respiratory tract
M64	226	II	−	Respiratory tract
M73	Diffuse	II	−	Respiratory tract
M70	<80	II	−	Respiratory tract
KL8	>860	II	−	Rheumatoid arthritis, joint
KL4	>860	II	−	Rheumatoid arthritis, joint
PG18	Diffuse	II	−	Human genital tract
12406	>1,100	II	−	Human genital tract
E10	160	I	+	Leukemic patient, blood
16700	160	I	+	Human genital tract
AOU	160	I	+	AIDS patient, blood
Z62	160	I	+	Leukemic patient, blood
Incognitus	160	I	+	Kaposi's sarcoma
AMSO	160	I	+	Lymphocyte culture
MT2	160	I	+	Human lymphocyte
Elliman	160	I	+	Hybridoma
DEPB	160	I	+	AIDS patient, blood
48429	160	I	+	Cell culture
K7	160	I	+	Leukemic patient, blood

[a] See Materials and Methods for descriptions.
[b] PCR was performed with the RS primers shown in Fig. 4.
[c] Southern blotting was performed with ORF-1 and ORF-2 probes.
[d] Immunoblotting was performed with MAb 1A2.6. −, negative; +, positive.

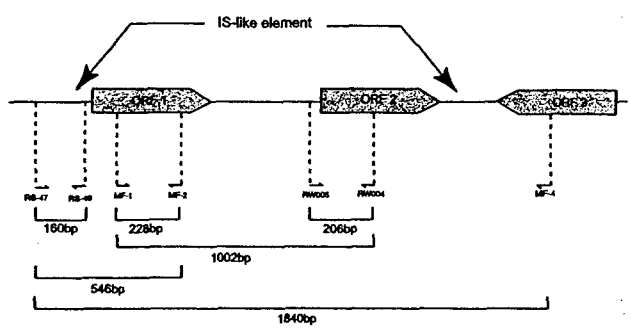

FIG. 2. Schematic diagram of the *M. fermentans* IS-like element and its flanking regions. The locations of all of the primer pairs used in this study are indicated, as are the sizes, in base pairs, of the respective amplicons. ORF-2 is the putative transposase. ORF-1 and ORF-3 have no assigned putative functions.

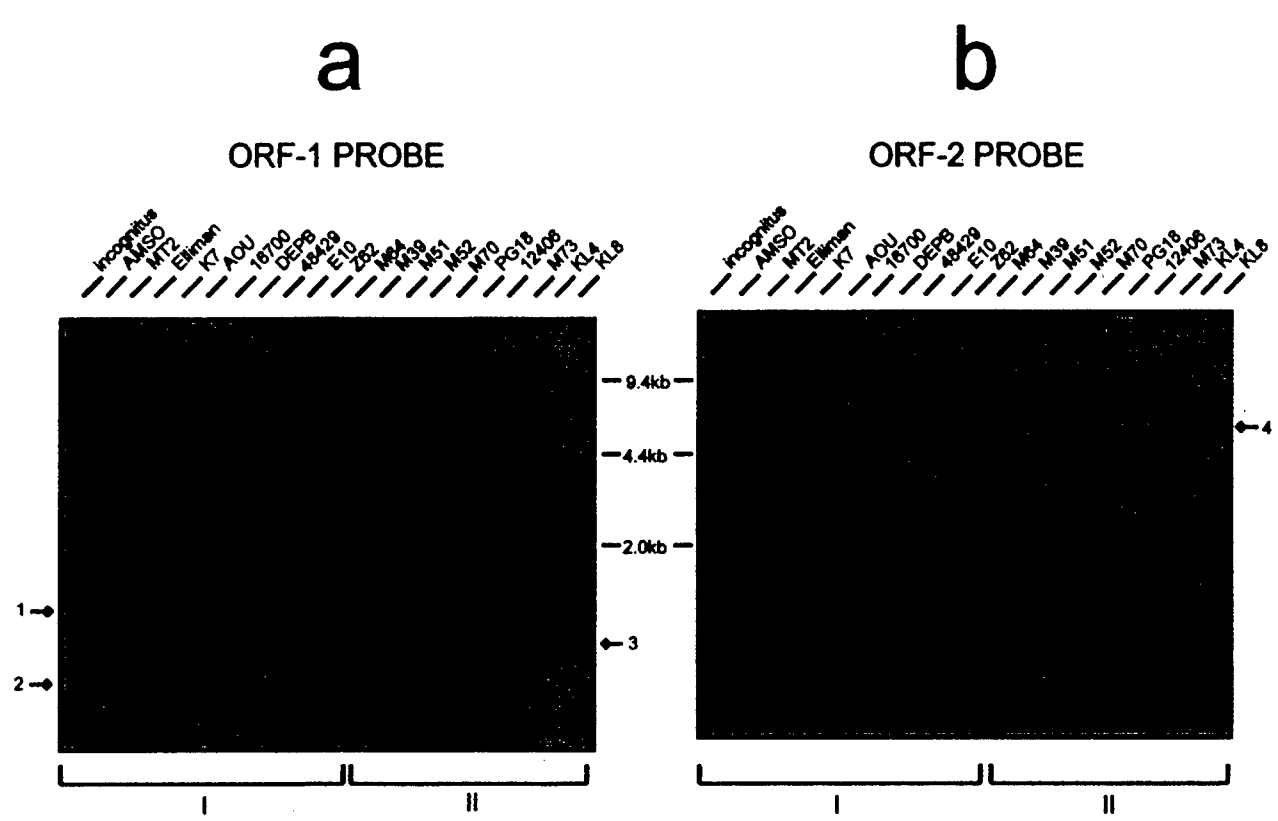

FIG. 3. Southern blot analysis of the chromosomal distribution of the *M. fermentans* IS-like element. DNAs isolated from all of the strains were digested with *Hin*dIII, separated in 0.8% agarose, transferred to nylon membranes, and then probed. The Southern blots were hybridized with an oligonucleotide specific for ORF-1 (a), and the same strains were then hybridized with an oligonucleotide specific for the transposase gene (b). Approximate fragment sizes are indicated in kilobases. Differences between the two basic genotypes (I and II) and the identification of fragments common to the two panels are indicated by the numbered arrows (see text).

60°C for 1 h and then boiled for 10 min. Samples were incubated on ice for 10 min and then stored at −70°C until ready for PCR.

Amplification of the *M. fermentans* strains was performed by using four different primer pairs (see Fig. 2). Primers RS-47 and RS-49 and primers RW005 and RW004 were previously described by S.-C. Lo et al. (34); primers MF-1 (5′-GGA AAA CTC TTA TTC AGC C-3′) and MF-2 (5′-GGA AAA CTC TTA TTC AGC ATG C-3′) were synthesized by Keystone Laboratories. Amplification of DNA was performed in a total volume of 50 μl. Basically, PCR was performed with 40 cycles of denaturation (94°C, 25 to 30 s), annealing (60°C, 1 min), and extension (72°C, 1 min). Another primer, MF-4 (5′-GCG GCA CCA TCA ATC ACA TAT AC-3′), was used as the antisense primer along with the previously described RS-47 sense primer. For this primer pair, an initial denaturation at 94°C for 2 min was followed by 40 cycles as described above. PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining.

Immunoblotting. SDS-polyacrylamide gel electrophoresis and Western blot analysis were performed as described previously, by using a 10% resolving gel and a 4% stacking gel, and then proteins were separated and transferred to nitrocellulose (Bio-Rad) by the method of Towbin et al. (33). Immunological reactions were visualized with peroxidase-labeled conjugates (Sigma).

MAbs. MAbs directed to *M. fermentans* incognitus antigens were produced in conjunction with the Hybridoma Core Facility of the Multipurpose Arthritis Center at the University of Alabama at Birmingham. The basic procedure for the production and characterization of MAbs has been described previously in detail (35).

RESULTS AND DISCUSSION

Phenotyping of *M. fermentans* strains and isolates. The most frequently colonized sites in a mycoplasma-infected host are epithelial cell surfaces (7, 12, 14, 23, 31). In the case of *M. fermentans*, the second most frequent association is with blood cells (17, 21). Are there characteristics that make some species or some strains within a species uniquely qualified for survival in one site as opposed to another? We previously identified an *M. fermentans* surface antigen (Pra) that can be divided into two distinct domains based on the immunoblot pattern obtained with MAbs, i.e., a domain that is resistant to degradation by trypsin, chymotrypsin, V-8 protease, and proteinase K and a second domain that is sensitive to these same proteinases (40). Our preliminary studies suggest that Pra is a complex surface network consisting of acylated proteins, but the nature of the membrane anchor and the noncovalent forces that mediate the interaction between the two domains have not been fully characterized (39). Nonetheless, we hypothesized that a correlation exists between the expression of the proteinase-resistant domain, which may play a protective role at the organism surface, and the association of the organism with particular cell types. Results in Fig. 1 show the variable expression of the Pra^+ phenotype and the distinctive, diffuse immunoblot pattern of the Pra^+ phenotype which correlated with isolation of the organism associated with cells of bone marrow origin and frequently from immunocompromised patients (Table 1). This broad distribution of the electrophoretic mobility of identical epitopes seen for the Pra^+ phenotype is not uncommon for mycoplasmal antigens (38). The single exception to the above correlation was isolate 16700, which was isolated from the urethra of a patient with nongonococcal urethritis. Organisms lacking expression of the proteinase-resistant phenotype were most commonly isolated from the respiratory tract, from the genital tract, and from arthritic joints (Fig. 1 and Table 1). These Pra mutants were presumably epithelial cell associated. If the Pra^+ phenotype does, in fact, provide the organism with protection from proteolytic degradation, then the above correlations support the possibility, although they certainly do not prove, that the Pra^+ phenotype resides in a hydrolase-rich

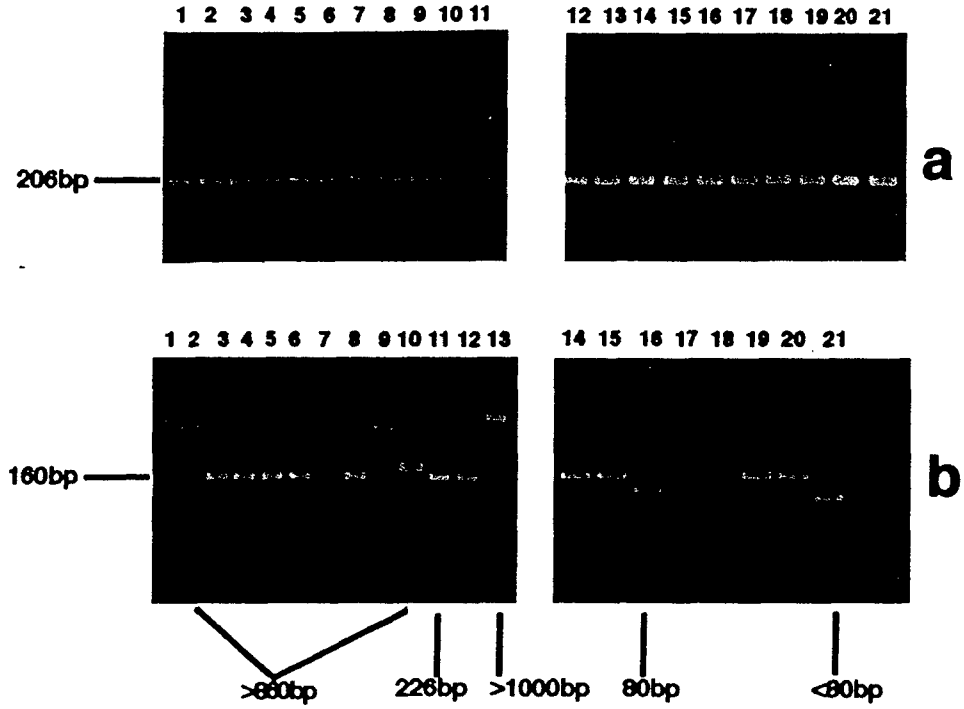

FIG. 4. PCR analysis of representative *M. fermentans* strains from various sources (Table 1), using primers RW005 and RW004 (a) and primers RS47 and RS49 (b), which are located within the transposase gene and upstream of the IS-like element, respectively (Fig. 2). PCR amplicons were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide. Sizes of products are indicated. Lanes: 1, M51; 2, KL8; 3, AMSO; 4, Elliman; 5, MT2; 6, DEPB; 7, M52; 8, AOU; 9, KL4; 10, M64; 11, E10; 12, 16700; 13, 12406; 14, Z62; 15, incognitus; 16, M39; 17, M73; 18, PG18; 19, K7; 20, 48429; 21, M70.

intracellular compartment. This niche may be represented by the professional-phagocyte-rich cellular environment found in the circulation.

Genomic distribution of the IS element. Development of a statistically sound proof of the association suggested above requires a large number of isolates characterized with respect to Pra expression and sites of isolation. Therefore, if a genetic marker also existed (preferably associated with Pra in a non-dissociable genotype-phenotype relationship) which correlated with the site of isolation, then it might be possible to develop a more sensitive and simple system for discerning the Pra phenotype of new isolates. We initially looked for a correlation between Pra expression and the genomic location of the previously published *M. fermentans* IS-like element. Disruption or activation of cryptic promoters is not an uncommon result of IS movement (1, 4). Although it is unproved, *M. fermentans* may use this system to accommodate its surface properties for survival in its current environment (13). Figure 2 is a schematic showing the basic structure of the previously described IS-like element, as well as the locations of the genotyping PCR primers and probes used in the current study. Figure 3 shows the distribution of IS-associated ORF-1 and ORF-2 (transposase gene) in *Hin*dIII genomic digests of the different *M. fermentans* strains. These restriction patterns are consistent with those previously shown for strain incognitus (13). Based on the distribution of these two ORFs, the *M. fermentans* strains can be grouped into two basic genotypes (I and II). The published sequence of the incognitus strain IS-like element contains no *Hin*dIII site (13). If the IS-like elements in the other *M. fermentans* strains are sufficiently similar to the IS-like element of strain incognitus and are intact, then ORF-1- and ORF-2-specific probes should cohybridize to the same *Hin*dIII restriction fragments. Examination of Fig. 3 by using this criterion (i.e., identification of fragments common to Fig. 3a and b) indicates that genotype I has at least 10 copies of the intact IS and genotype II has only 3. These are minimal estimates, since a single fragment could contain multiple probe sites. Identification of fragments that are not common to Fig. 3a and b indicate that (i) all genotype I strains have one copy of ORF-1 which is not associated with an IS (Fig. 3a, location 1); (ii) two of the genotype I strains, incognitus and 16700, have one additional non-IS-associated ORF-1 (Fig. 3a, location 2); (iii) 7 of the 10 genotype II strains have one non-IS-associated ORF-1 (Fig. 3a, location 3); and (iv) 6 of the latter 7 genotype II strains also have a non-IS-associated ORF-2 (Fig. 3b, location 4). The apparent non-IS-associated ORFs in these experiments may be the result of a common mutational event producing a *Hin*dIII site between ORF-1 and ORF-2. Strains KL4 and KL8 are unusual, since no IS elements are detectable in Fig. 3. Even though the latter two strains may actually constitute a third genotype, here we have placed them into genotype II based on additional parameters to be discussed below.

Table 1 shows genotypes I and II in the context of Pra expression and site of isolation. All strains in genotype I were Pra^+, and all genotype II strains were Pra mutants.

Genotyping of *M. fermentans* strains by PCR. The pattern of distribution of the IS-like element genomic insertion sites was a reliable reflection of Pra expression but required very cumbersome methodology. The asymmetric hybridization of the ORF-1 and ORF-2 probes seen in Fig. 3 indicated the presence of some differences within the IS-like element which may provide useful sequence markers for determining genotypes by using PCR. An *M. fermentans*-specific PCR primer pair has been previously described (34). Amplification with this primer pair, located within ORF-2 of the IS-like element (Fig. 2, RW005 and RW004), resulted in a 206-bp amplicon for the

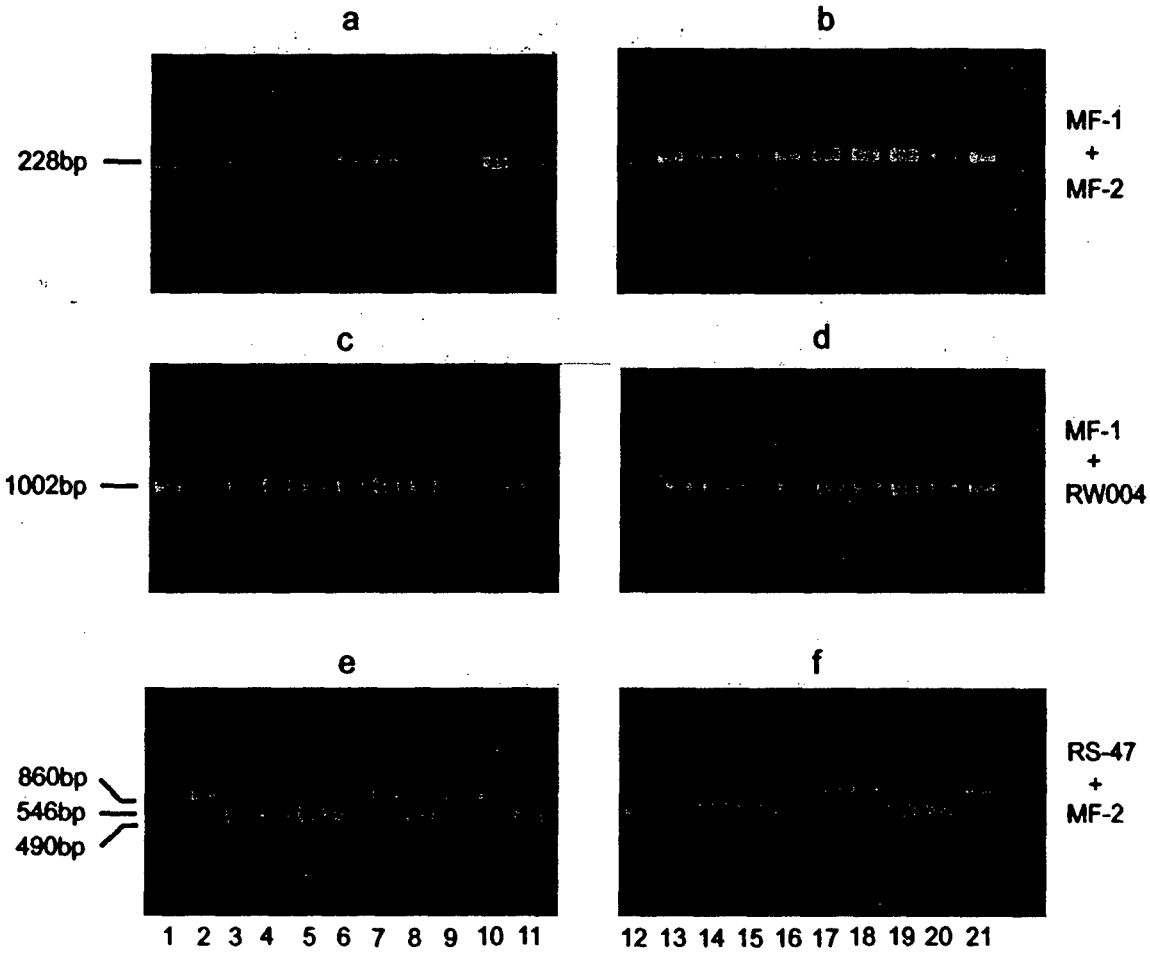

FIG. 5. PCR analysis of *M. fermentans* strains. Amplification of representative *M. fermentans* strains from various sources (Table 1) by using primers MF-1 and MF-2, MF-1 and RW004, and RS-47 and MF-2 (Fig. 2) is shown. PCR amplicons were analyzed by electrophoresis in 2% agarose gels and stained with ethidium bromide. Lanes in a, c, and e: 1, M51; 2, KL8; 3, AMSO; 4, Elliman; 5, MT2; 6, DEPB; 7, M52; 8, AOU; 9, KL4; 10, M64; 11, E10. Lanes in b, d, and f: 12, 16700; 13, 12406; 14, Z62; 15, incognitus; 16, M39; 17, M73; 18, PG18; 19, K7; 20, 48429; 21, M70. Sizes of products are indicated on the left.

strains evaluated in that study. In the current study, amplification with these same primers produced a 206-bp amplicon for all of the strains listed in Table 1 (Fig. 4a). Although these primers performed as predicted, they do not allow discrimination of the two genotypes. Strains KL4 and KL8 showed no indication for the presence of either ORF-1 or ORF-2 (Fig. 3), even though a typical 206-bp product was obtained with the RW005-RW004 primer pair (Fig. 4a). This suggested that even though the RW005 and RW004 primer sites were present, the intervening sequence was sufficiently different to disallow hybridization of the RW006 probe. This may result in a dysfunctional transposase, which could explain the absence of multiple insertion sites in these two strains.

In the same previous study as that described above, another primer pair located immediately upstream of the IS-like element (Fig. 2, RS47 and RS49) produced a 160-bp product for only three of the six strains tested. The RS47-RS49 primer pair was not further evaluated, since those investigators were interested in defining PCR primers for detection of all strains of *M. fermentans*. When we evaluated the strains listed in Table 1 with the RS47-RS49 primer pair, the most common product was 160 bp (Fig. 4b). Other products ranged from ≤80 bp (M39 and M70) to over ≥860 bp (KL8, KL4, and 12406). Some strains also gave no distinct product (M51, M52, M73, and PG18). These results are recorded in Table 1, and there is a complete correlation between genotype I, Pra expression, and the IS-like element location resulting in a 160-bp product with the RS primers. PCR amplification of all genotype II strains resulted in different-sized amplicons or no amplicon. The lack of an RS product for strains M51, M52, M73, and PG18 implies that either one or both primer sites are missing (or lack sufficient homology) or that there is an insertion between the primer sites resulting in a template which was too large to amplify efficiently.

Various PCR primer combinations (Fig. 2) were used to determine if there were any differences in the sequence within the IS-like element which might allow the detection of a stable PCR product that would be representative of the genotype II strains. Amplification with the primers MF-1 and MF-2 suggested that ORF-1 was not significantly different among the strains (Fig. 5a and b). Strains KL4 and KL8 gave no product, and strain 16700 consistently produced a weak amplicon. Similarly, by using primers MF-1 and RW004, we found no differences in the linkage between ORF-1 and ORF-2 among the strains (Fig. 5c and d). Once again, strains KL4 and KL8 gave no product and strain 16700 was amplified poorly. The linkage between the RS genomic site and the ORF-1 IS site was evaluated by using primers RS-47 and MF-2 (Fig. 5e and f). Am-

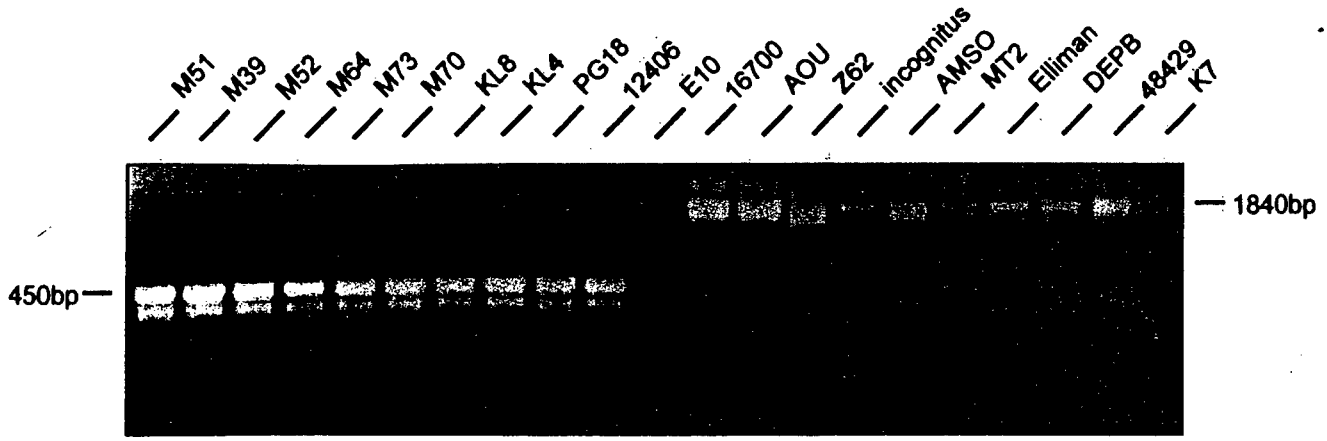

FIG. 6. PCR analysis of the *M. fermentans* strains by using primer sites which flank the IS-like element. Primer pair RS-47–MF-4 (Fig. 2) amplicons were analyzed by electrophoresis in a 2% agarose gel and stained with ethidium bromide. Sizes of products are indicated. Genotype II strains are represented by the 450-bp product. Genotype I strains were not consistently amplified.

plification of all genotype I strains resulted in a 546-bp amplicon, reaffirming the stability of this particular insertion site for the genotype I strains. The genotype II strains were inconsistently amplified with this primer pair, indicating the instability of this insertion site in these strains.

Since Fig. 4b, 5e, and 5f suggested that the genotype II strains do not have an IS-like element consistently present near the RS site, we used primers RS-47 and MF-4, which flank the putative insertion site, to ascertain if the IS was consistently absent from this site. Figure 6 shows a stable 450-bp product for all genotype II strains, indicating that the genotype II strains have a stable linkage between the RS site and ORF-3 with no intervening IS-like element.

These analyses indicate that the two *M. fermentans* genotypes consistently differed in the sites of insertion of the IS-like element but not in the sequence of the element itself. Also, due to potential sequence instability within the IS-like element, the only reliable genotyping markers reside outside the IS-like element and in the genomic sequences immediately upstream and downstream of the IS junctions. Therefore, the method of choice for species detection appears to be direct detection of specific sequences within the IS-like element. In contrast, for genotype distinction, detection of stable genomic insertion sites is required, i.e., with primers RS47 and RS49 for genotype I and primers RS47 and MF-4 for genotype II. These primer pairs should allow easy and rapid genotyping of *M. fermentans* in clinical samples and thus obviate the need for the frequently unsuccessful isolation and culturing of this organism.

Aside from the immediate usefulness for diagnostic genotyping, these data also will help to understand how this species may adapt for survival in a particular host population. At this time, we have only a suggestive link between the sites of insertion of the IS-like element and Pra expression, and as previously stated by Lo et al. (13), there is no proof that this element is mobile. Conclusive evidence will have to await a more direct connection between the *pra* gene sequence and a specific insertion site. We have not analyzed a sufficient number of isolates to say that proteinase resistance is always associated with isolation from cells of a blood-related compartment or from immunocompromised patients, but completion of these analyses will determine if *M. fermentans* uses the mobility of this IS-like element coupled to Pra expression as a primary means of maintaining a specific niche in its host.

ACKNOWLEDGMENTS

This research was supported by grant DAMD17-97-1-7001 from the Department of the Army and grant 5R01 AI33197 from the National Institutes of Health. MAbs were produced in conjunction with the Hybridoma Core Facility of the Multipurpose Arthritis Center at the University of Alabama at Birmingham, which is supported by grant 2P60 AR0614-20 from the National Institutes of Health.

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In Vitro **Immunological Measures Are Not Altered in Symptomatic Gulf War Veterans**

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Abstract

Context The underlying etiology and pathogenesis of Gulf War veterans' illnesses continue to be under intense investigation. Reports have suggested the basis for these illnesses may be an altered immune system, but compelling evidence is lacking.

Objective To determine whether *in vitro* immune responses are abnormal in symptomatic Gulf War veterans versus controls

Design, Setting, and Participants A randomized case-control study conducted by blinded comparison of laboratory measures of *in vitro* immune responses in blood samples obtained from veterans in an outpatient facility of a Veterans Affairs medical center. Symptomatic Gulf War veterans with otherwise undefined illnesses (31 symptomatic veterans), asymptomatic Gulf War veterans (31 asymptomatic controls), and veterans who had applied for disability compensation and had not participated in the Gulf War (21 disability controls) represented the volunteer sample.

Main Outcome Measures *In vitro* cellular and humoral immune responses; to detect functional abnormalities in antigen presenting cells (autologous mixed leukocyte reactions and expression of interleukin (IL)-1β, IL-6, IL-10, and tumor necrosis factor-α); T cells (lymphocyte proliferation using the polyclonal T-cell activators phytohemagglutinin and Concanavalin A, primary immune responses in allogeneic mixed leukocyte reactions, and secondary immune response using the recall antigens tetanus toxoid, *Candida albicans*, and anthrax vaccine); type-1 T-helper cells (gamma interferon expression); type-2 T-helper cells (IL-4 and IL-10 expression); and B cells (polyclonal B-cell activator pokeweed mitogen-induced immunoglobulin production).

Results In general, immune response measures did not differ significantly between groups. Heightened responses observed in the disability control group (sporadically greater responses to one mitogen and two antigens) and the Gulf War participation control group (greater recall responses to anthrax vaccine) did not suggest impaired immune cell function in symptomatic veterans when compared with controls.

Conclusion *In vitro* immunological responses are not abnormal in symptomatic Gulf War veterans.

Introduction

Approximately 697,000 United States military service members were deployed to the Persian Gulf to participate in Operation Desert Shield/Desert Storm during the latter months of 1990 and early months of 1991. After returning from the Gulf War, many veterans began reporting various symptoms that they thought were associated with military service during the Gulf War.[1] These symptoms included fatigue, musculoskeletal complaints, cognitive dysfunction, headaches, gastrointestinal distress, skin problems, and sleep disturbances and apparently were referable to multiple organ systems.

The underlying etiology and pathogenesis of these symptoms continues to be under intense investigation. A number of articles have suggested that immunological disorders or immune dysfunction may have developed in Gulf War veterans as a result of Gulf War participation. Most of these reports are review or hypothesis articles and do not provide supportive data.[2,4,5] In their hypothesis article, Rook and Zumla[2] speculate that "Gulf War syndrome" may be due to a systemic shift in cytokine balance towards a T-helper 2 (Th2) profile, i.e., with Th2 cytokines such as interleukin (IL)-4 and IL-10 expressed more predominantly than Th1 cytokines such as IL-2 and gamma interferon. The balance of cytokine expression is important in maintaining proper immune function since Th2 cytokines are known to inhibit the synthesis of Th1 cytokines and suppress cell-mediated immunity, whereas Th1 cytokines can down-regulate antibody production on the humoral side of the immune system.[3] These authors suggest that "Gulf War syndrome" may represent a special case of chronic fatigue syndrome since patients with chronic fatigue can have low production of Th1 cytokines. They also raise the possibility that multiple vaccinations (with characteristics that favor Th2-induction) might be the stimulus that leads to a Th2-dominant cytokine balance. Korenyi-Both and colleagues[4] have made vague assertions that microimpregnated sand particles in the Gulf War "depleted the immune system." Ferguson and Cassaday[5] suggest that "Gulf War syndrome" may be an IL-1–mediated sickness response and thus due to elevated levels of IL-1, but have provided no supportive data.

Zhang and coworkers[6] reported that service in the Persian Gulf is associated with an altered immune status in veterans who also returned with a severe fatiguing illness. They found that veterans with chronic fatigue syndrome had significantly higher levels of IL-2 and gamma interferon (as well as IL-10 and tumor necrosis factor-alpha) than the controls. This study is compatible with that proposal by Rook and Zumla, however, the data reveal elevated levels of Th-1 cytokines instead of reduced levels that would have been expected by Rook and Zumla's

theory. In contrast, Soetekouw and colleagues[7] found no shift in cytokine balance, i.e., not even towards a Th2 profile, in Dutch soldiers with symptoms of fatigue and memory loss who had participated in the UNTAC (United Nations Transitional Authority in Cambodia) peacekeeping operation during 1992-93. Similarly, Klaustermeyer and colleagues[8] performed extensive laboratory testing and immediate and delayed hypersensitivity skin testing and showed that Gulf War veterans with a multitude of nonspecific symptoms had no immune abnormalities detected with these tests. -

Since these available studies are inconclusive regarding the issue of detectable immunological abnormalities in symptomatic Gulf War veterans, we undertook a systematic evaluation of different components of the acquired immune response system in symptomatic Gulf War veterans compared with those of asymptomatic Gulf War veterans and disability controls. Since previous reports and hypotheses do not indicate that Gulf War veterans were prone to develop recurrent or opportunistic infections, if an immunological abnormality were responsible for their symptoms, it would appear that it would reside in the acquired rather than innate immune system. Therefore, we used sensitive *in vitro* immunological assays in efforts to detect functional abnormalities in antigen presenting cells, including dendritic cells and monocytes; T cells; Th1 and Th2 cells; and B cells that function in acquired immunity. This study was undertaken to determine if Gulf War veterans have significant immunological abnormalities due to service in the Gulf War.

Methods

Subjects and Controls: After the Gulf War, the Department of Veterans Affairs (VA) developed a Gulf War registry protocol examination that was implemented at most VA medical centers for those deployed to the Persian Gulf during Operation Desert Shield/Desert Storm (i.e., Gulf War participation from August 1990 to March 1991)[1]. Demographic information was collected and veterans were offered physical examinations. At the initiation of this study, the Birmingham VA Medical Center had 1,550 individuals in its registry. VA medical records were requested on each, and 1,342 records were available for review. A registered nurse previously trained in chart abstraction reviewed each chart and compiled a listing of all symptoms and diagnoses listed in the medical records and, when available, onset of the symptoms. To confirm reliability of the abstraction process, the first twenty charts were also reviewed by a physician to determine if the process was complete and accurate. Based on the chart reviews, 193 (14.4%) were asymptomatic. A physician (WDB) then reviewed the abstracted data from the

remaining 1149 charts. If questions arose from the abstracted data, the physician then reviewed the medical record. Patients were excluded if the listed diagnoses explained the ongoing symptoms or if the onset of symptoms antedated the Gulf War. Veterans excluded because of an established diagnosis also resulted in exclusion of those treated with medications such as corticosteroids or immunosuppressives that might have altered *ex vivo* immune function testing. The physician then ascribed the recorded symptoms to one or more of the following organs systems: dermatological, genitourinary, pulmonary, cardiovascular, neurological, ear/nose/throat, gastrointestinal, or musculoskeletal. For symptoms that could be attributed to several organ systems, such as shortness of breath, a clinical decision was made based on the treating physician's opinion, if documented in the record, or lacking that, based on the complex of symptoms recorded in the record. Patients were further excluded if their symptoms were attributable to only one organ system. This left 177 veterans whose records indicated that they had symptoms attributable to at least two organ systems that were otherwise unexplained. Efforts were made by phone and/or mail to contact all 177 symptomatic Gulf War veterans for participation in laboratory studies. Informed consent and samples were obtained from 52 of these symptomatic veterans (symptomatic group).

The first control group (asymptomatic group) included the 193 asymptomatic veterans from the Birmingham registry. An attempt was made to contact each of them, again by either phone or mail. Thirty one agreed to enter the study.

The second control group was from a group of veterans who were not involved in the Gulf War but had applied for disability compensation at the Birmingham VA Medical Center at the time of the study. This disability control group (disability group) was established by contacting the Alabama Department of Veterans Affairs and matching disability claims for veterans with medical records on file at the Birmingham VA Medical Center. This match yielded 321 medical records for veterans. Attempts were made to contact all 321 veterans, however, only 21 veterans were found who volunteered to provide laboratory samples.

Attempts were made by phone and/or mail to contact all veterans in all three of the subject and control groups for study participation. There was no a priori attempt to match for any demographic characteristic in any group. When study participants were contacted for venipuncture, demographic and lifestyle information was obtained. Veterans from the asymptomatic group were also questioned to assure they remained asymptomatic.

Body mass index was calculated for veterans based upon their height and weight reported at the time of deployment and at the time of patient enrollment.

Laboratory samples and measurements: Study participants had venous blood drawn in the morning into tubes containing sodium heparin to yield blood cells for preparation of peripheral blood mononuclear cells (PBMCs) and plasma for cytokine testing. The next procedure was rigorously followed in efforts to decrease variation in cytokine levels caused by inadvertent monocyte activation during processing[94] or proteolysis of cytokines by contaminating platelets. Freshly drawn blood samples were placed immediately on ice and delivered to the processing laboratory within 30 minutes of collection. Samples were centrifuged at 4°C to yield red and white blood cell-free plasma followed by platelet removal from plasma by centrifugation in a high-speed, micro centrifuge for 2 minutes. Plasma was aliquoted into 1.0-ml aliquots and frozen at -70°C until assayed. Cytokine analyses were performed in batch determinations to decrease inter-assay variation. These conditions appear optimal for cytokine determinations.[9-11]

PBMC were prepared and enriched by centrifuging blood diluted 50:50 (v/v) in RPMI 1640 (Gibco Co, Grand Island, NY) over Ficoll for 20 min at 400 X g. PBMC were collected, washed twice with RPMI 1640, counted, resuspended in culture medium (CM) consisting of RPMI 1640 supplemented with 5% human AB serum (ICN, Costa Mesa, CA), gentamicin (20 μg/ml; Sigma Co., St. Louis, MO), and L-glutamine (2 mM; Sigma) and adjusted to a concentration of 8 X 10^6 PBMC/ml.

Immunological testing was done on Gulf War veteran and control blood samples using the following *in vitro* immunological assays in efforts to detect functional abnormalities in antigen presenting cells including dendritic cells and monocytes (autologous mixed leukocyte reactions and expression of interleukin (IL)-1β, IL-6, IL-10, and tumor necrosis factor-alpha [TNF-α]); T cells (lymphocyte proliferative capacity using the polyclonal T-cell activators phytohemagglutinin [PHA] and Concanavalin A [Con A], primary immune responses in allogeneic mixed leukocyte reactions, and secondary immune response using the recall antigens tetanus toxoid, *Candida albicans* [*C. albicans*], and anthrax vaccine); type-1 T-helper (Th1) cells (gamma interferon [IFN-γ] expression); type-2 T-helper (Th2) cells (IL-4 and IL-10 expression); and B cells (polyclonal B-cell activator pokeweed mitogen-induced immunoglobulin [Ig] production).

Blood was collected and processed on 104 subjects and controls. Immune function tests for mitogen- and antigen-induced lymphocyte proliferation and mixed leukocyte reactions were attempted on all subjects; however, *n* values less than 104 indicate that the volume of blood was not sufficient for all tests to be performed on a given patient(s), the PBMC yield was not sufficient on a given patient(s), or a lab error resulted in no reportable data for a

given patient(s). Samples (blood plasma and/or stimulated cell supernatants) for pokeweed mitogen-induced Ig production and cytokine expression were stored at -70°C prior to batch analysis.

For all stimulation or proliferation cultures, PBMC were cultured in triplicate in CM in flat-bottom, 96-well tissue culture plates at 37°C in a humidified, 5% CO_2 atmosphere. For proliferative cultures induced by graded doses of the mitogens PHA (Sigma) and Con A (Sigma), PBMC (2 X 10^6 PBMC/ml, 50 µl/well) were cultured for 72 hr. For proliferative cultures stimulated by the antigens tetanus toxoid (Wyeth-Ayerst Laboratories Inc., Marietta, PA) and *C. albicans* (Bayer Co., Spokane, WA), PBMC (2 X 10^6 PBMC/ml, 50 µl/well) were cultured for 7 days. PHA was used at 4.0, 2.0, and 1.0 µg/ml final concentration; Con A was used at 10.0, 5.0, and 2.5 µg/ml final concentration; tetanus toxoid was used at 5.0, 2.5, and 1.25 % (v/v) final concentration; and *C. albicans* was used at 1.25, 0.625, and 0.31 protein nitrogen units/ml final concentration. To test for recall responses to anthrax vaccine, purified recombinant protective antigen was used as the antigenic stimulator in proliferative cultures at empirically derived doses of 14.4, 7.2, and 3.6 µg/ml final concentration. This protective antigen, a protein which is the protective component of the vaccine, was derived from an asporogenic strain of *Bacillus anthracis* that was engineered to express the protective antigen (according to personnel at the United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD, who provided this antigen).[12]

To inhibit growth of pooled allogeneic stimulator PBMCs that were used in allogeneic mixed leukocyte reactions, cells at 5 X 10^6/ml were mixed with mitomycin C (Sigma) to yield 40µg/ml final concentration in CM. Cells were incubated at 37°C for 50 min. on a rotator and washed 4 times. Stimulators were diluted to 4, 2, and 1 X 10^6/ml and added to microculture plates at 50 µl/well. Responder PBMCs (2 X 10^6/ml) were added to wells in 50 µl volumes. Cultures were incubated for 6 days at 37°C/5% CO_2 in a humidified incubator. Autologous mixed leukocyte reactions were performed in a similar fashion by adding 50 µl/well of PBMC from each patient at 4, 2, and 1 X 10^6 cells/ml to wells of tissue culture plates in the absence of exogenous stimulators.

For all proliferative assays, cultures were pulsed during the final 4 hours of culture with 1.0 µCi ^{3}H-thymidine (Amersham Pharmacia Biotech, Buckinghamshire, UK) and harvested on a semi-automated cell harvester (Skatron Instruments, Inc., Sterling, VA). Thymidine incorporation was used as an indicator of cellular proliferation, assessed on a beta scintillation counter (LS Analyzer model 6000 IC; Beckman Instruments, Inc., Norcross, GA), and recorded as counts per minute. Cellular proliferation was recorded as stimulation index: mean counts per minute incorporated into cells in the presence of antigen or mitogen divided by mean counts per minute incorporated

into cells in the absence of antigen or mitogen (medium alone). An exception to this was in autologous mixed leukocyte reactions where medium without added cells (medium control) was used as the divisor to calculate stimulation indices. Results are reported as mean stimulation index ± standard error (SE).

For cytokine generation, PBMC (2 X 10^6/ml, 50 µl/well) were mixed with PHA at 5 µg/ml final concentration and incubated for 24 hr. Supernatants were harvested and pooled for each patient. Duplicate determinations of cytokine and other analyte levels were made using enzyme-linked immunosorbent assay (ELISA) kits purchased from R&D Systems (Minneapolis, MN) for IL-1β, IL-4, IL-6, IL-10, IFN-γ, TNF-α, and soluble intercellular adhesion molecule-1 (sICAM-1) as previously described.[13,14] Soluble IL-2 receptor (sIL-2R) ELISA kits were purchased from Endogen (Woburn, MA).

To measure Ig production, 2 X 10^6 PBMC were placed into 10-ml tubes containing 2 ml of either 2.5 µg/ml pokeweed mitogen to assess pokeweed mitogen-induced Ig production or medium for spontaneous Ig production. After a 7-day incubation, cell-free supernatants were harvested and frozen at -70°C until assayed for IgM, IgG, and IgA production by isotype-specific ELISA following the ELISA kit manufacturer's (Bethyl Laboratories, Inc., Montgomery, TX) protocol.

Laboratory and demographic data for study groups were statistically compared using analysis of variance and post-hoc analysis for pairwise comparisons of three groups: symptomatic, asymptomatic, and disability. Categorical data comparisons were performed using the chi square or Fisher exact test when appropriate. Our sample size was such that parametric procedures were sufficient for all analyses of continuous data. Also descriptives (means, SE, and frequencies) were generated for all relevant variables.

Results

Demographic characteristics of the three study groups (including age, sex, race, years of military service, rank, battlefield position in Gulf War, type of military service, and years of formal education) are listed in Table 1. Overall, the disability controls were younger than the Gulf War veterans. The asymptomatic Gulf War veterans had more years of military service than the disability control group, but were not different from the symptomatic group. There was also a modest but statistically significant difference in terms of years of education between the two Gulf War veteran groups. None of the disability control group was from the military Reserves or National Guard. When compared with asymptomatic controls, symptomatic Gulf War veterans were less likely to be Caucasian (Caucasian

incidence: 58% versus 77%), had 4 years less military service (18 versus 22 years), were more likely to be enlisted rather than officers (enlisted-to-officer ratio: 89% versus 79%), and were more likely to report participation in the Gulf War on military front lines versus military support positions (43% versus 33%), but none of these differences reached statistical significance. Differences between these groups and the disability group were also reported and are depicted in Table 1.

Information regarding medical and social history was collected (including height and weight at time of deployment to the Persian Gulf, weight at time of study enrollment, use of alcohol and tobacco products, and whether the veterans had received an anthrax or tetanus vaccination) and are listed in Table 2. Asymptomatic veterans were taller than the symptomatic veterans at time of deployment. As would be expected, the disability group who did not deploy to the Persian Gulf and receive vaccinations while overseas had a lower percentage of veterans who reported that they had received an anthrax vaccination(s) compared with the Gulf War veterans. Symptomatic Gulf War veterans were less likely than asymptomatic veterans to use alcohol (57% versus 72%) and more likely to use tobacco products (57% versus 38%).

As noted in the methods section, the symptoms documented in the medical records were reviewed by a physician and ascribed to organ systems. A comparison of the organ systems involved in the sample of 52 symptomatic Gulf War veterans and the remaining 125 veterans in the original population of 177 symptomatic Gulf War veterans from which the sample was derived revealed that there were no significant differences between the groups. These results are shown in Table 3. On average, each symptomatic Gulf War veterans who volunteered (n=52) had symptoms attributable to 4.87 organ systems and those who did not volunteer (n=125) had symptoms attributable to 4.79 organ systems. The most common symptoms appeared to be related to, in descending order, neurological, musculoskeletal, and gastrointestinal systems. These observations indicate that the sampled group was representative of the original population of symptomatic veterans.

Laboratory results

Overall, no abnormalities were detected that would indicate that any of the study groups were immunodeficient or immunocompromised. Studies were designed to evaluate the functional integrity of cells involved in acquired immunity. Because acquired immune responses are complex in nature, several approaches were used in this evaluation. Sensitive *in vitro* immunological tests were done in efforts to detect any functional

abnormalities in cells involved in acquired immunity: antigen presenting cells, including dendritic cells and monocytes, were evaluated using autologous mixed leukocyte reactions (a sensitive measure of functional integrity of both dendritic cells and T cells)[15,16] and expression of interleukin (IL)-1β, IL-6, IL-10, and tumor necrosis factor-alpha [TNF-α]; T cells were evaluated by measuring lymphocyte proliferative capacity using the polyclonal T-cell activators phytohemagglutinin [PHA] and Concanavalin A [Con A], primary immune responses in allogeneic mixed leukocyte reactions, and secondary immune response using the recall antigens tetanus toxoid, *Candida albicans* [*C. albicans*], and anthrax vaccine; type-1 T-helper (Th1) cells were evaluated by testing for gamma interferon [IFN-γ] expression; type-2 T-helper (Th2) cells were evaluated by testing for IL-4 and IL-10 expression; and B cells were evaluated by measuring polyclonal B-cell activator pokeweed mitogen-induced immunoglobulin [Ig] production.

Secondary or memory immune responses (i.e., T-cell memory) were measured using the common recall antigens tetanus toxoid and *C. albicans*. Immune memory was also tested for a recall antigen with which some veterans were vaccinated while in the United States or during their Gulf War tour of duty, i.e., via anthrax vaccinations. The measure for T-cell function in secondary immune responses was antigen-induced T-cell proliferation. Pokeweed mitogen-induced Ig production was used as a measure of T-cell–dependent B-cell function.

Analysis of experimental data from peripheral blood mononuclear cell (PBMC) cultures stimulated with T-cell mitogens revealed that PHA-activated T-cell proliferation did not differ significantly among the study groups (Table 4). However, statistically significant differences were noted in the Con A-induced proliferative responses. Closer inspection of the data in Table 4 revealed that the differences for Con A studies were seen chiefly in the disability group. However, these differences were not noted throughout the dose response range for Con A and were not noted in PHA responses. These data indicate that there are not substantial differences in these three groups with regard to T cell capacity to respond to plant mitogens suggesting that T cell function is not substantially impaired in any group.

PBMC cultures were also analyzed using autologous and allogeneic mixed leukocyte reactions as well as antigen-induced proliferative responses and Ig production. These mixed leukocyte reactions did not differ significantly among the study groups (Table 4) indicating that dendritic cell and T-cell functions in primary *in vitro* immune responses are not abnormal in these groups. However, statistically significant differences were noted in the antigen-induced proliferation cultures. Analysis of the data in Table 4 revealed that the differences for antigen-induced responses were not different across the dose response ranges tested, but instead revealed sporadic

differences. In the tetanus toxoid and *C. albicans* cultures, the differences were chiefly due to increased responsiveness in the disability control group. Antigen-induced proliferative assays were also performed using the weak immunogen found in anthrax vaccine. Statistically significant differences were found more often in proliferative responses to anthrax vaccine than in the other two antigen cultures. This was chiefly due to heightened responses in the asymptomatic group. The data for spontaneous immunoglobulin production of IgM, IgG, and IgA by PBMC and that induced using the polyclonal B-cell activator pokeweed mitogen did not vary significantly between groups (not shown).

To test different immune cells for their capacity to express cytokines important for cellular immunity and Ig production, plasma and stimulated mononuclear cell supernatants were analyzed for patterns of cytokine expression (Table 5). Unstimulated PBMC were also tested for cytokine expression to determine if abnormal steady-state levels were present in cells of symptomatic Gulf War veterans versus control values. Determination of patterns of cytokine expression might be suggestive of an altered or skewed immune response and would reveal if functional abnormalities were present in antigen presenting cells, type-1 T-helper cells (Th1), and type-2 T-helper cells (Th2). Specifically, interleukin (IL)-1β, IL6, IL-10, and tumor necrosis factor-alpha (TNF-α); gamma interferon (IFN-γ); and IL-4 and IL-10 levels were used as measures of monocyte, Th1, and Th2 activation, respectively. Levels of soluble factors associated with fever, weight loss, and chronic inflammatory diseases, i.e., IL-6, TNF-α, soluble IL-2 receptor (sIL-2R), and soluble intercellular adhesion molecule-1 (sICAM-1), were also analyzed in an effort to determine whether or not selected soluble markers could serve as markers of symptoms in Gulf War veterans.

Performance of cytokine determinations revealed that cytokine levels in the study groups were not statistically significantly different from one another for a given analyte. More specifically, plasma, unstimulated PBMC supernatants, and PHA-stimulated PBMC supernatants were tested for IL-1β, IL-4, IL-6, IL-10, IFN-γ, TNF-α, sIL-2R, and sICAM-1. Certain trends were noted, but these were not statistically significantly different. The only statistically significant difference noted was lower IFN-γ in unstimulated supernatants from symptomatic group.

Discussion

The results of this systematic evaluation of *ex vivo* measures of acquired immune responsiveness (including tests to detect functional abnormalities in antigen presenting cells, T cells, Th1 cells, Th2 cells, and B cells that are important in acquired immunity) in a group of patients with undiagnosed symptoms that developed after the Gulf War demonstrated no significant abnormalities.

Ferguson and Cassaday suggested that "Gulf War syndrome" may be an IL-1–mediated sickness response and thus due to elevated levels of IL-1.[5] They go on to suggest that those that have this "syndrome" should have elevated IL-1 levels in their blood compared to Gulf War veterans who are asymptomatic. However, our data for circulating plasma and mitogen-induced levels of IL-1β reveal no such statistically significant elevations in IL-1β values when symptomatic Gulf War veterans are compared with asymptomatic veterans and disability controls.

The minor but significant differences noted in T-cell responses for Con A, tetanus toxoid, and *C. albicans* could be due to differences in ages between the groups since heightened responses were noted for these variables for the disability group and this group is significantly younger than the Gulf War veterans in the symptomatic and asymptomatic groups. Moreover, it has been documented that mitogen- and antigen-induced immune responses decline with age.[17-19] However, there was no direct statistical effect of age on these laboratory results when regression analyses were performed.

The data from our study suggest that Gulf War participation did not negatively impact on the Gulf War veterans' capacity to mount an immune response to an antigen (i.e., anthrax vaccine) to which they had been exposed for the first time around the time of deployment to the Gulf War. This is important in that most of these veterans would have received their vaccination(s) with this antigen in close proximity to their time of deployment to the Gulf War, and had their immune systems been dampened or suppressed by Gulf War participation, it would have been expected that they would have a dampened recall response when tested *in vitro*.

Overall, the anthrax vaccine is a relatively weak immunogen and a course of vaccination is required.[20,21] We did find that responses to anthrax vaccine were greater in the asymptomatic Gulf War veterans when compared to the other two groups. Certainly these data do not provide any indication that there is an association between Gulf War symptoms and this finding, as our disability control group had responses similar to symptomatic Gulf War veterans. Unfortunately, our data do not provide an explanation for these differences. Documents regarding who actually received the full course or any of the anthrax vaccination are no longer available and we had to rely on self-reporting. Nonetheless, there were no statistically significant results when data from only those patients who

reported having received the anthrax vaccine were analyzed. Undetected differences in the prevalence of vaccination with this antigen between the groups could provide a simple explanation of this finding. In each group we found veterans whose cells did respond to anthrax even though the veteran reported no exposure, suggesting self-reporting of this information is not completely accurate.

One of the difficulties in studying symptomatic patients who were in the Gulf War arises from the fact that there has been no generally accepted case definition of "Gulf War Syndrome." After the war, there had been suggestions that "Gulf War syndrome" is a unique new disorder. Therefore, we chose to select patients from our registry who had been evaluated and had symptoms that their treating physicians could not ascribe to a particular diagnosis. Charts were carefully scrutinized by the study nurse and findings reviewed by the study physician. We did exclude patients whose symptoms related to only one organ system, as this did not seem to reflect the spectrum of symptoms being reported that are purported to be related to Gulf War involvement. Many of these patients reported symptoms such as evanescent rashes that providers had not been able to observe.

Other investigators have noted difficulties in recruiting veterans to enroll in Gulf War studies. Of our 177 symptomatic Gulf War veterans who had their charts reviewed, we were only able to enroll 52 veterans (29%). Since the ones we did study had no major alterations in their *ex vivo* immunologic responsiveness, we think it unlikely that there is sampling bias. However, we did obtain demographic and lifestyle information from those who did not volunteer to come to the center for venipuncture. There were no significant demographic or lifestyle differences between those we did study and the 125 veterans we did not. Moreover, the symptoms in the 125 veterans mapped to organ systems in the same distribution as those we did study, suggesting that the studied group is indeed representative of the population.

Obviously this study has been completed nearly a decade after the Gulf War. However, the patients we studied remained symptomatic, and despite this, no immunologic abnormalities were noted.

Patients thought to have disorders such as asthma, chronic bronchitis, post-traumatic stress disorder, or fibromyalgia were excluded. Other studies of Gulf War veterans have included patients with post-traumatic stress disorder or fibromyalgia and our results do not address these groups. However, these patients from other settings with post-traumatic stress disorder or fibromyalgia have had similar *ex vivo* immunological evaluations and support for an immunological component in the pathogenesis of these disorders is limited. In light of these observations and

the paucity of other reported significant abnormalities, Gulf War veterans can be reassured that there are no significant immunological abnormalities due to service in the Gulf War.

Conclusions

The underlying etiology and pathogenesis of unexplained illnesses in Gulf War veterans continue to be under intense investigation, and some reports have suggested the basis for these illnesses may be an altered immune system. However, compelling evidence for such suggestions is lacking. We therefore performed studies to determine whether *in vitro* immune responses are abnormal in symptomatic Gulf War veterans versus asymptomatic Gulf War veteran and symptomatic non-Gulf War veteran controls. Results of sensitive *in vitro* cellular and humoral immune responses used to detect functional abnormalities in antigen presenting cells, T cells, and B cells revealed no consistent differences between study groups and indicated that *in vitro* immunological responses are not abnormal in Gulf War veterans. These data should reassure Gulf War veterans that there are no significant immunological abnormalities due to service in the Gulf War.

Acknowledgements

This study was supported by funds from the U.S. Army Medical Research Acquisition Activity for the DoD project DoD-42.

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Tables.

Table 1. Demographic Characteristics of Symptomatic and Asymptomatic Gulf War Veterans and Disability Controls			
		Study Groups	
	Symptomatic	**Asymptomatic**	**Disability**
Characteristics	**(n=52)**	**(n=31)**	**(n=21)**
Age in 1997,			
mean (SE), y	45.4 (1.6)	**47 (2.2)***	**39.8 (2.5)***
Male, %	88.5	90.3	81.0
Race/ethnicity,			
Distribution, %			
Caucasian	57.7	77.4	61.9
African American	42.3	22.6	33.3
Other	0	0	4.8
Service, mean (SE), y	17.9 (1.5)	**22.3 (1.9)***	**12.1 (2.6)***
Enlisted, %	88.5	79.3	93.8
Front lines, %	43 (7.0)	33.0 (9.0)	15 (13.0)
Guard/Reserve, %	**68.0***	**70.0***	**0***
Formal education,			
Mean (SE), y	**13.2 (0.2)***	**14.1 (0.3)***	14.1 (0.4)
*Significantly different ($p < 0.05$); Age: Asymptomatic vs. Disability: p=0.032;			
Service: Asymptomatic vs. Disability: p=0.002; Guard/Reserve: Disability vs.			
Symptomatic & Asymptomatic: ≤ 0.001; Education: Symptomatic vs. Asymptomatic: p=0.028.			

Table 2. Medical and Social History of Symptomatic and Asymptomatic Gulf War Veterans and Disability Controls			

		Study Groups	
	Symptomatic	**Asymptomatic**	**Disability**
Characteristics	**(n=52)**	**(n=31)**	**(n=21)**
Height at deployment,			
mean (SE), m	**1.674* (0.015)**	**1.730* (0.020)**	1.692 (0.025)
Weight at deployment,			
mean (SE), kg	78.52 (2.00)	82.78 (2.54)	77.93 (4.08)
BMI† at deployment	28.02	27.67	27.23
Weight at study enrollment,			
mean (SE), kg	83.78 (2.22)	89.36 (2.95)	83.24 (3.95)
BMI at study enrollment	29.90	29.87	29.09
ETOH use (%)	56.9	72.4	43.8
Tobacco products use (%)	56.9	37.9	62.5
Anthrax vaccine received (%)	**62.8***	**68.0***	**21.4***
Tetanus vaccine received (%)	87.0	92.6	92.3
*Significantly different ($p < 0.05$); Height at deployment: Symptomatic vs. Asymptomatic: p=0.025.			
Anthrax vaccine received: Disability vs. Symptomatic & Asymptomatic: p=0.011.			
†Body Mass Index			

Table 3. Comparison of symptoms ascribed to various organ systems in 177 chart-reviewed symptomatic Gulf War veterans

	Volunteers*	**Non-Volunteers**
	n=52	**n=125**
Organ System	**%**	**%**
Neurological	17.8	19.0
Musculoskeletal	17.0	16.2
Gastrointestinal	14.2	14.2

Pulmonary	14.2	12.2
Dermatological	11.9	12.5
Cardiovascular	4.0	3.2
Ear/Nose/Throat	12.6	13.0
Genitourinary	5.1	5.5

*52 Gulf War veterans volunteered for study participation

Table 4. *In vitro* Immune System Functional Tests of Symptomatic and Asymptomatic Gulf War Veterans and Disability Controls

		Study Groups	
	Symptomatic	**Asymptomatic**	**Disability**
Test	**mean (SE)**	**mean (SE)**	**mean (SE)**
Mitogen-induced T-cell proliferation:			
Con A, µg/ml:	N=45	n=29	n=17
10	132 (15.9)	110 (18.6)	162 (24.3)
5	93.4 (11.5)	**80.6*** (14.4)	**131*** (18.8)
2.5	**61.5*** (8.7)	**51.0*** (10.8)	**95.9*** (14.2)
PHA, µg/ml:†	N=19	n=12	n=13
4	309.4 (24.9)	269.1 (31.3)	283.4 (30.0)
2	237.7 (20.7)	190.4 (26.1)	212.1 (25.1)
1	121.8 (15.0)	93.6 (18.9)	102.6 (18.2)
Autologous and allogeneic mixed leukocyte reactions:			
Autologous, PBMC/ml:	N=43	n=28	n=17
4 X 10^6	4.4 (1.4)	2.7(1.7)	7.8 (2.3)
2 X 10^6	2.1 (0.4)	1.5 (0.5)	2,7 (0.6)
1 X 10^6	1.5 (0.2)	1.4 (0.2)	1.4 (0.2)
Allogeneic, PBMC/ml:‡	N=43	n=28	n=17

4 X 10^6	63.4 (14.5)	74.4 (18.0)	71.4 (23.0)
2 X 10^6	37.5 (8.7)	34 (11.0)	46 (13.0)
1 X 10^6	13.7 (4.1)	13.6 (5.1)	25.6 (6.6)
Antigen-induced T-cell proliferation:			
Tetanus toxoid, µg/ml:	N=42	n=29	n=17
5	35 (8.9)	29.1 (10.7)	66.6 (14.0)
2.5	30.2 (7.4)	**22.1*** (8.9)	**50.6*** (11.6)
1.25	29.8 (7.8)	27.2 (9.4)	54.5 (12.3)
Anthrax vaccine, µg/ml	N=20	n=12	n=11
14.4	1.35 (0.40)	2.18 (0.51)	1.37 (0.53)
7.2	**1.26*** (0.45)	**3.40*** (0.61)	**1.31*** (0.61)
3.6	**1.26*** (0.47)	**3.29*** (0.63)	**1.16*** (0.63)
C. Albicans, U/ml§	N=34	n=28	n=17
1.25	12.4 (2.9)	5.1 (3.2)	14.4 (4.2)
0.625	13.0 (2.9)	7.0 (3.2)	17.1 (4.2)
0.031	12.3 (2.9)	**5.2*** (3.2)	**18.1*** (4.1)
*significant differences, p <0.05 **(in bold)**: Con A (5 µg/ml): Asymptomatic vs. Disability: p=0.033;			
(2.5 µg/ml): Symptomatic vs. Disability: p=0.041, Asymptomatic vs. Disability: p=0.014.			
Tetanus toxoid (2.5 µg/ml): Asymptomatic vs. Disability: p=0.036.			
Anthrax vaccine (7.2 µg/ml): Symptomatic vs Asymptomatic: p=0.007, Disability vs. Asymptomatic:			
p=0.019; (3.6 µg/ml): Symptomatic vs. Asymptomatic: p=0.015; Disability vs. Asymptomatic: p=0.022.			
C. albicans (0.31 U/ml): Asymptomatic vs. Disability: p=0.015.			
†Dose responses on remainder of samples was done with a different batch of PHA and also did			
not yield significant differences.			
‡These values represent numbers of mitomycin C-treated pooled allogeneic stimulator cells			
co-cultured with 2 x 10^6 subject or control PBMC responders			
§Protein nitrogen units			

Table 5. Levels of Expression of Cytokines and Soluble Factors in Supernatants and Plasma in Gulf War Veterans and Disability Controls			
		Study Groups	
	Symptomatic,	**Asymptomatic,**	**Disability,**
Tests	**Mean (SE)**	**mean (SE)**	**mean (SE)**
Unstimulated supernatant:			
IL-1β, pg/ml	64.1(21.3)	66.4 (27.5)	56.5 (34.8)
IL-4, pg/ml	ND*	ND	ND
IL-6, pg/ml	4520 (1562)	4849 (2017)	7359 (2552)
IL-10, pg/ml	ND	ND	ND
IFN-γ, pg/ml	**5.9† (2.1)**	14.79 (2.7)	13.1 (3.5)
TNF-α, pg/ml	211.2 (56.7)	175.8 (73.2)	194.8 (92.6)
Stimulated supernatant:			
IL-1β, pg/ml	909 (115.3)	656.1 (148.8)	795.6 (188.3)
IL-4, pg/ml	58 (7.2)	64.7 (9.3)	63.8 (11.8)
IL-6, pg/ml	24871 (2338)	26429 (3019)	30982 (3819)
IL-10, pg/ml	208 (19.8)	179 (25.6)	174 (32.3)
IFN-γ, pg/ml	1739 (192)	1081 (248)	1521 (314)
TNF-α, pg/ml	3048 (226)	2549 (291)	3167 (369)
Plasma:			
IL-1β, pg/ml	BDL‡	BDL	BDL
IL-6, pg/ml	1.9 (0.6)	2.21 (0.81)	3 (0.73)
TNF-α, pg/ml	0.72 (0.25)	1.26 (0.34)	0.77 (0.31)
sIL-2R, pg/ml	5107 (571)	4212 (766)	5610 (647)
sICAM-1, ng/ml	157 (23.5)	234 (31.5)	221 (26.6)
*Not done due to levels below detection noted in preliminary samples			

†Significantly different; IFN-γ for Symptomatic vs. Disability: p=0.013.			
‡ Below Detectable Limit			

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 27 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the enclosed list of technical documents. Request the limited distribution statement assigned to the documents listed be changed to "Approved for public release; distribution unlimited." These documents should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
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